AD

HOST AND ENVIRONMENTAL FACTORS INFLUENCING THE MANIFESTATION AND PROPAGATION OF THE YEAST PRIONS

FINAL TECHNICAL REPORT

BY

MICHAEL D. TER-AVANESYAN

MAY 2002 - JULY 2003

UNITED STATES ARMY

EUROPEAN RESEARCH OFFICE OF THE U.S. ARMY

LONDON, ENGLAND

RAD 9263

CONTRACT NUMBER N62558-02-M-5868

INSTITUTE OF EXPERIMENTAL CARDIOLOGY,
CARDIOLOGY RESEARCH CENTER,
MOSCOW, RUSSIA

APPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED

20030910 086

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	6 June 2003	Final Report, 14 May 02 - 14 July 03

4. TITLE AND SUBTITLE
Host Factors Influencing Propagation and Manifestation of the Yeast [*PSI*$^+$] Prion

5. FUNDING NUMBERS
C-62558-02-M-5868

6. AUTHOR(S)
Michael D. Ter-Avanesyan

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Institute of Experimental Cardiology, Cardiology Research Center, 3rd Cherepkovskaya Street, 15A, Moscow 121552, Russia

8. PERFORMING ORGANIZATION REPORT NUMBER
Final Report

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
US Naval Regional Contracting Center, Detachment London Government Buildings, Block 2, Wing 12 Lime Grove, Ruislip, Middlesex HA4 8BX, United Kingdom

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The artificial prion [PSI$^+$] based on a hybrid Sup35PS with prion domain from the yeast Pichia methanolica [PSI$^+_{PS}$] was used to search a multicopy Saccharomyces cerevisiae genomic library for novel factors able to cure prions. It was found that overexpression of the Hsp40 family chaperones Sis1 and Ynl077w, chaperone Sti1, transcriptional factors Sfl1 and Ssn8 and acidic ribosomal protein Rpp0 interfere with propagation and manifestation of the [PSI$^+$] prion. This implies that similar factors may be active against human and animal prion and amyloid diseases.

A novel electrophoretically-based approach for studying the structure of the Sup35 prion particles was developed. This method was used to show that Sup35 prion aggregates accumulated in the [PSI$^+$] cells represent agglomerations of small amyloid-like polymers, whose size is characteristic of a given [PSI$^+$] variant. Inactivation of the Hsp104 chaperone by guanidine HCl causes rapid and reversible increase in the size of such Sup35 polymers indicating essential role of the chaperone Hsp104 in prion polymer fragmentation. The approach developed may be useful for the analysis of other prion and amyloid proteins and especially the mammalian prion, PrP.

14. SUBJECT TERMS
Electrophoresis, chaperones, ribosomal protein, transcriptional factors, translation termination, yeast prions

15. NUMBER OF PAGES 23

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT

TABLE OF CONTENTS

1. A STATEMENT OF THE PROBLEM

The ability of some proteins to undergo autocatalytic conformational alterations represents a common molecular basis for incurable amyloid and prion diseases in man and animals. The progress in understanding the molecular mechanisms of these diseases was accelerated recently by the finding that similar processes occur in lower eukaryotes being manifested as phenotypic alterations independent of DNA or RNA, but related to conformational changes of certain proteins. In the yeast Saccharomyces cerevisiae, several protein-based genetic elements, propagating by a mechanism postulated earlier for mammalian prion diseases were identified, which allowed to call them yeast prions. In this project a specially developed genetic system based on the yeast SUP35 gene, encoding the eRF3 (Sup35p) translation termination factor, was employed to study the structure of yeast prion particles and genetic and environmental factors influencing prion propagation and manifestation. Such data may allow to develop approaches for the treatment of human prion and amyloid diseases.

2. BACKGROWND AND SIGNIFICANCE

2.1. Mammalian prions

Recent years were distinguished by the appearance and rapid development of a fundamentally novel area in the molecular biology and medicine: the prions and prion-like proteins. Such proteins can switch stably into an alternative functional state, which allows them to act as infectious agents or as heritable genetic elements. It is possible to anticipate that further progress in this area will have a great impact on both biology and medicine.

Prions are a unique class of infectious agents, whose infectivity is related solely to protein. In mammals, they cause fatal neurodegenerative diseases, such as Creutzfeld-Jacob disease of man, sheep scrapie and bovine spongiform encephalopathy (BSE) [for review, see 1, 2]. Epizootic of BSE in Britain led to a sharp increase of the interest to prion diseases especially because recent data showed a danger of prion infection transfer to humans by consumption of meat from animals with prion diseases.

The prion infectious process relies on the presence of host-encoded prion protein (PrP), which is able to exist in two structurally and functionally different forms. Of them, the infectious form (PrP^{Sc}) can catalyze conversion of the normal cellular form (PrP^{C}) into the PrP^{Sc}. Due to this autocatalytic nature, the prion conversion proceeds as a chain reaction and can result in the conversion of most of PrP^{C} into PrP^{Sc}. Furthermore, PrP^{Sc} is highly resistant to proteases, in contrast to PrP^{C}. This causes PrP^{Sc} accumulation, which could be a major cause of the neuronal death. The difference in properties of PrP^{C} and PrP^{Sc} results from the conformational alteration of PrP^{Sc}, rather than from any covalent modifications. PrP^{Sc} is rich in β-sheet, while PrP^{C} is almost devoid of it. It should be noted that all mammalian prion diseases are related to a single protein, PrP. However, it is highly important that the prions could be just a part of a much wider phenomenon. We discuss below that two other phenomena, amyloid diseases and yeast prions are based on the same principles as the prions. Despite somewhat different manifestations, these phenomena are united by a unique mechanism of autocatalytic conformational rearrangement of proteins.

Amyloid diseases represent a group of about 20 human diseases, which are characterized by deposition in different tissues of fibrous aggregates of conformationally altered proteins [for review, see 3]. Some of these diseases, like Alzheimer's and Parkinson's disease represent a major challenge for the public health care in the developed countries. Although amyloidogenic proteins are structurally and functionally unrelated, they form very similar amyloid fibers, which are about 10 nm in width and have a characteristic cross-β structure, in which the individual β strands are oriented perpendicular to the axis of fiber. Amyloids can bind Congo red dye, giving a characteristic birefringence in polarized light. There is a number of significant similarities between the prion and amyloid phenomena. Prion diseases are often accompanied by fibrous PrP deposits, which show the characteristic properties of amyloids: an increased β sheet content and the ability to bind Congo red. In turn, amyloid fibers conform to the criteria for prion protein: the normal and polymer forms of amyloidogenic proteins are structurally different and the latter can promote the polymerization of normal proteins into amyloid fibers.

There are two competing models for the process of the prion conversion, which represent different understanding of the structure of prions. The heterodimer model assumes that prion, existing as a monomer, can form a heterodimer complexes with normal molecules catalyzing their prion rearrangement. Then two molecules dissociate and the cycle repeats. According to this model, the oligomerization is a secondary process, inessential for the prion conversion [4] The nucleated polymerization model is universal and describes the formation of both prions and amyloids. This model considers them as regular polymers, or one- dimensional crystals that serve as nuclei for further polymerization of the protein. The conformational rearrangement of monomers may either occur spontaneously and then be fixed in the polymer structure, as originally suggested [5], or, alternatively, be directly catalyzed by polymer at the moment of

accretion [1]. Recently the latter model was substantially modified. Several important observations indicated that prion conformation is adopted concomitantly with assembly, via molten oligomeric intermediates [6]. What is important, however, is that the prion and amyloid phenomena are similar by the polymerization models, but different by the heterodimer model. Making a choice between these models is essential for understanding of the prion and amyloid phenomena.

2.2. Prions of lower eukaryotes

The analogy with prions allowed to explain the unusual properties of two yeast genetic determinants, [PSI^+] and [URE3] [7], and the [Het-s] determinant of the filamentous fungus Podospora anserina [8]. These determinants show a non-Mendelian mode of inheritance. However, unlike other cytoplasmic factors, such as mitochondrial genes and viruses, they may be eliminated by growth in the presence of low concentrations of protein denaturant, guanidine hydrochloride (GuHCl), and can reappear without introduction of new DNA, upon overexpression of proteins responsible for maintenance of these factors. The most studied among these determinants is the yeast [PSI^+] determinant, which causes suppression of nonsense mutations. To explain the unusual genetic behavior of the [PSI^+] determinant, it has been suggested that Sup35p, which is the translation termination factor eRF3 [9, 10], may use the prion-like autocatalytic mechanism to switch into an alternative conformational and functional state, resulting in the [PSI^+] phenotype. This suggestion, originally based on genetic evidence, has quickly gained a biochemical support. It was demonstrated that the properties of Sup35p in [PSI^+] cells ($Sup35p^{PSI+}$) are altered and similar to that of PrP^{Sc}: $Sup35p^{PSI+}$ shows increased protease resistance and is found mostly in aggregated state [11, 12]. The prion conversion of Sup35p was reproduced in vitro, and it was demonstrated that highly purified $Sup35p^{PSI+}$ is able to initiate such conversion efficiently [13]. The chain of sequential conversion reactions was performed using an aliquot of the previous reaction to start the next one, thus modeling in vitro the continuous propagation of the Sup35p prion state. Another very important observation was that Sup35p purified from [PSI^+] cells could assemble into uniform fibers, which showed characteristic features of amyloids [14, 15]. These findings demonstrated that yeast prions are related to both prions and amyloidogenic proteins of mammals, which supports the idea that the prion and amyloid phenomena are related [for review, see 16, 17]. Recently it was shown that fibers formed in vitro by the Sup35 protein purified from Escherichia coli can be used to transform yeast cells from the [psi^-] to the [PSI^+] state [18]. This experiment represents the most direct proof of the prion concept.

Yeast prions provide numerous advantages as a model system for studying the prion and amyloid phenomena. For yeast, a wide range of different methods of analysis is now available, including powerful methods of classic genetic analysis and recombinant DNA techniques. Yeast prions are safe for men. All these factors allowed the rapid progress in studies of yeast prions: during a short period the key prion properties were demonstrated for yeast prion-like proteins and some important novel mechanisms were discovered. The further use of yeast system for studying the prion phenomenon will help to understand the molecular mechanism of prion conversion of proteins and how widespread is the prion phenomenon in nature, to decipher the molecular mechanisms of "protein only" inheritance and its biological significance. They can shed light on molecular basis of prion diseases as well as of other amyloidoses and may help to find strategies to treat these diseases.

3. RESULTS AND DISCUSSION

The proposed project was planned to be performed in three years (May 14 2002 - May 13 2005). This final report covers the first year of the grant term (May 14 2002 - July 13 2003). During the first year of the project accomplishment our efforts were mainly directed to the achievement of the following aims:

1. Finding of novel factors influencing prion propagation in yeast.
2. Development of a novel biochemical approach for studying the structure of prion particles.

3.1. Novel factors influencing prion propagation in yeast

While in vitro Sup35 alone is sufficient for its conformational rearrangement, in vivo some cellular proteins can participate in this process and modulate its efficiency. The most important of them is the Hsp104 chaperone, which is strictly required for the [PSI^+] propagation, although its excess interferes with [PSI^+] [19]. The chaperones of Hsp70 family showed weaker effects: the overexpression of Ssb1 cured "weak" [PSI^+] strains [20, 21], while overproduction of Ssa1 interfered with the [PSI^+] curing by excess Hsp104 [22].

Construction of heterologous [PSI^+] ([PSI^+_{PS}]) provided a sensitive instrument for identification of novel factors involved in the prion propagation. [PSI^+_{PS}] is based on a hybrid Sup35, in which the native prion domain was replaced with its analog from the yeast Pichia methanolica [23]. Compared to [PSI^+], [PSI^+_{PS}] showed increased sensitivity to

overexpression of the Hsp70 chaperones Ssa1, Ssb1 and the Hsp40 chaperone Ydj1, but decreased sensitivity to the excess of Hsp104 [21]. The [PSI^+_{PS}] curing by chaperones showed remarkable prion strain specificity. The chaperones ranked differently by their curing efficiency for each prion strain. This finding supports the existence of different prion structures corresponding to prion strains and differential sensitivity of these structures to curing mechanisms associated with different chaperones. Thus, the sets of chaperones able to cure a given prion may differ for different prions and prion strains. Recent studies of another yeast prion, [RNQ^+], showed that it depends on a chaperone of the Hsp40 family, Sis1, which has not been found among the factors curing [PSI^+] [24]. Deletion of inessential glycine and phenylalanine-rich region of Sis1 abolished propagation of this prion. It should be noted that all mentioned chaperones are considered to be functionally related. Hsp70 and Hsp40 represent eukaryotic homologues of the bacterial chaperone system DnaK-DnaJ and Ydj1 and Sis1 are presumed to be partners for Ssa1 [25]. In vitro, Ssa1 and Ydj1 cooperated with Hsp104 to reactivate the aggregates of heat-denatured luciferase, with each of the chaperones being essential for this process [26].

To find additional factors, which can influence the prion propagation in yeast and presumably in other organisms, we decided to use the potential of the [PSI^+_{PS}] system to perform a systematic screen for the genes, which cure [PSI^+_{PS}] when overexpressed.

A screen for prion-eliminating factors - The strain 5V-H19 (MATa ade2-1 SUQ5 ura3-52 leu2-3,112 can1-100 [psi^-]) and its derivative, PS-5V-H19 obtained from 5V-H19 by replacing SUP35 for the chimerical SUP35-PS allele, were used in this work. Both strains carry the ade2-1 UAA nonsense mutation and the SUQ5 (SUP16) UAA nonsense suppressor, but differ from each other by the SUP35 gene. The original strain carries the wild-type SUP35 gene allowing maintenance of the conventional [PSI^+], while its derivative possesses the hybrid allele of this gene, SUP35-PS, necessary for propagation of [PSI^+_{PS}] [23]. SUQ5 suppresses ade2-1 only in combination with [PSI^+] or [PSI^+_{PS}], which results in adenine prototrophy and white colonies. The cells lacking these determinants are adenine requiring and form red colonies. The strain PS-5V-H19 [$PSI^+_{PS\text{-}1}$] was transformed with two S. cerevisiae genomic libraries based on the multicopy plasmids YEplac195 and pRS426. A total of about 300 000 transformants were screened. Expression of plasmid-encoded factors interfering with the [PSI^+_{PS}] maintenance should either cure [PSI^+_{PS}] or slow down the prion conversion, causing increased levels of soluble Sup35. Transformants with such plasmids should have red, red sectored or pink colonies, in contrast to white colonies of other transformants. About 500 such transformants were selected and refined by cloning.

To discriminate the antisuppression and [PSI^+_{PS}] curing from spontaneous [PSI^+_{PS}] loss, two clones of each transformant were crossed to a tester 1A-H74 [$PSI^+_{PS\text{-}1}$] strain. In this test, the transformants that had lost [$PSI^+_{PS\text{-}1}$] spontaneously should produce white [PSI^+_{PS}] diploids. Such transformants were discarded. Fifty transformants produced red or pink color in diploids and were used for plasmid DNA isolation. The plasmids were used to transform the PS-5V-H19 [$PSI^+_{PS\text{-}1}$] strain, and 24 of them yielded red, red sectored or pink transformants. These plasmids were sequenced from the ends of genomic inserts, and the inserts were identified by comparison of the sequences with the yeast genome. Since the inserts usually contained several genes, the genes affecting [PSI^+_{PS}] were identified by deletion analysis. The following genes were found: SIS1 (6 clones, 2 independent plasmids), YNL077w (3, 1), STI1 (1, 1), SFL1 (2, 1), SSN8 (3, 1), RPP0 (3, 1), SUP35 (6, 3). The SUP35 gene product, Sup35, causes antisuppression, but not [PSI^+_{PS}] loss, since Sup35 does not aggregate in [PSI^+_{PS}] cells and does not interfere with Sup35PS prion aggregation [23]. The YNL077w gene encodes a previously uncharacterized protein of the Hsp40 (DnaJ) family designated hereafter as Apj1 (Anti-Prion DnaJ). The other genes encode Hsp40 chaperone Sis1, chaperone Sti1, transcriptional factors Sfl1 and Ssn8, and acidic ribosomal protein Rpp0.

The effects of overproduction of these proteins, the antisuppression and prion loss, were characterized for the independently isolated strains of hybrid and conventional [PSI^+] (Table 1). For [$PSI^+_{PS\text{-}1}$], only excess Sis1 showed significant antisuppression, while excess Rpp0 caused the highest prion loss, but low antisuppression. It should be noted that, as it was observed previously [21], the relative efficiency of chaperones in prion curing varied depending on the [PSI^+] strain and the origin of Sup35 prion domain.

TABLE 1. Effects of the "anti-prion" factors. The effects were determined for the transformants of indicated [PSI$^+$] strains overproducing described proteins. Control, transformants with the YEplac195 vector. The loss of [PSI$^+$] or [PSI$^+_{PS}$] was scored as a ratio of red and white colored colonies. "Sectored loss" reflects a visual proportion of red sectors in colonies. ++, strong effect, +, weak effect, -, no effect.

[PSI$^+$] Strain	Effect	Overproduced proteins							
		Sis1	Apj1	Ydj1	Sti1	Sfl1p	Ssn8p	Rpp0	Control
[PSI$^+_{PS-1}$]	Antisuppression	++	+	+	+	+	+	+	-
	Sectored loss	-	-	+	-	-	-	++	-
	% loss	11	9	12	12	3	0	25	0
[PSI$^+_{PS-2}$]	Antisuppression	+	+	+	-	+	+	-	-
	Sectored loss	-	-	-	-	-	-	-	-
	% loss	10	22	5	2	5	2	0	0
[PSI^+_S]	Antisuppression	-	-	-	-	+	+	-	-
	Sectored loss	-	-	-	-	-	-	-	-
	% loss	0	0	0	0	3	5	0	0
[PSI^+_W]	Antisuppression	-	-	-	+	+	+	-	-
	Sectored loss	-	-	-	-	+	-	-	-
	% loss	0	0	0	2	15	3	0	0

Sfl1 binds the heat shock element - Transcriptional response to heat shock is mediated by binding of Hsf1 to the heat shock element (HSE) typical of heat-inducible promoters [27]. The similarity of Sfl1 to Hsf1 suggests that Sfl1 could also bind to HSE and direct the expression of heat shock-related proteins. Such possibility was studied by mobility shift assay. HSE was synthesized as an oligonucleotide duplex of 25 base pairs. Purified Sfl1 was obtained via E. coli expression. Labeled HSE was incubated with Sfl1 or yeast lysates differing in the levels of Sfl1 and run on a gel (Fig. 1). HSE showed an efficient binding to pure Sfl1, which suggests that Sfl1 can prime the HSE-dependent transcription. HSE also bound some components of yeast lysates, and the amount of such complexes correlated with the Sfl1 levels. This suggests that Sfl1 was a component of these complexes, together with some other proteins, since the mobility of the complexes was lower than that of the pure Sfl1-HSE complex. The amount of the HSE-protein complexes in the lysates lacking Sfl1 was reduced greatly respectively to the wild-type lysates. This suggests that under the experimental conditions Sfl1 was a major protein bound to HSE. In contrast, pure Sfl1 did not bind to STRE (data not shown), another DNA element typical of many heat- and stress-inducible promoters [27]. Nevertheless, the amount of protein bound to STRE in the lysates notably depended on the Sfl1 levels, being the lowest in the wild-type lysates. This allows to suggest that although Sfl1 does not bind STRE, it exerts a significant indirect influence on the STRE-driven transcription (Fig. 1).

Overexpressed Rpp0 is found in ribosome-free fractions - Rpp0 represents one of the five so-called acidic ribosomal proteins, which form a stalk at the large subunit of a yeast ribosome. Among them, Rpp0 plays a key role, since it is essential for viability and mediates binding of four other acidic ribosomal proteins to the ribosome. Earlier, it was observed that the increased transcription of RPP0 did not lead to increased levels of Rpp0 and its appearance free from ribosomes [28]. Such conclusion would exclude a possibility of any effects of multicopy RPP0. Therefore, we studied the levels of Rpp0 and its subcellular distribution. The immunoblot analysis of Rpp0 revealed an about 3-fold excess of it in the total extracts of cells with multicopy RPP0 (Fig. 2). The lysates were fractionated by centrifugation through a sucrose density gradient, and the ribosomal fractions were identified by the presence of ribosomal RNA. This allowed finding Rpp0 in sub-ribosomal fractions, though not in a free monomeric form. The amount of ribosome-free Rpp0 was rather low in a control lysate, and much higher in the one overexpressing Rpp0. Therefore, the multicopy expression of RPP0 led to the appearance of Rpp0 in the form of relatively large ribosome-free complexes.

Alterations of the Sfl1, Ssn8 and Rpp0 levels affect expression of chaperones - In our experiments, the prion curing was probably due to the altered levels of proteins directly involved in the prion propagation. The studied factors could either belong themselves to such proteins, or act indirectly, by altering expression of the proteins of this group. The first opportunity appears likely for the chaperones Sis1, Apj1 and Sti1, whose function is to mediate the protein folding. The second suits best for the transcriptional factors Sfl1 and Ssn8, as well as for the ribosomal protein Rpp0. Although Rpp0 is not a transcriptional factor, its influence on gene expression may be expected, since the lack of acidic ribosomal proteins Rpp1A/B and Rpp2A/B, structurally and functionally related to Rpp0, altered the levels of

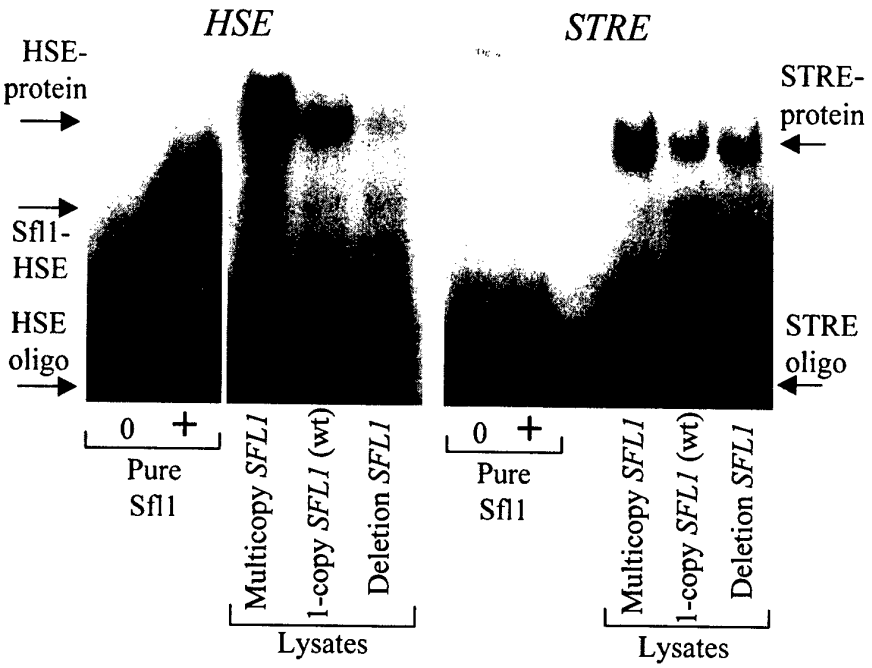

Figure 1. Sfl1 binds to the HSE, but not STRE DNA elements. Labeled HSE and STRE DNA fragments were incubated with either purified His_6-Sfl1 or with yeast lysates prepared from the PS-5V-H19 [*psi*$^-$] cells with the multicopy *SFL1* plasmid (m*SFL1*), Yeplac195 vector (control) or *SFL1* disruption (Δ*SFL1*), and analyzed on 4.5% polyacrylamide gel. The positions of HSE and STRE complexes with His_6-Sfl1 or lysate proteins are indicated by arrows. -, no His_6-Sfl1, +, His_6-Sfl1 added.

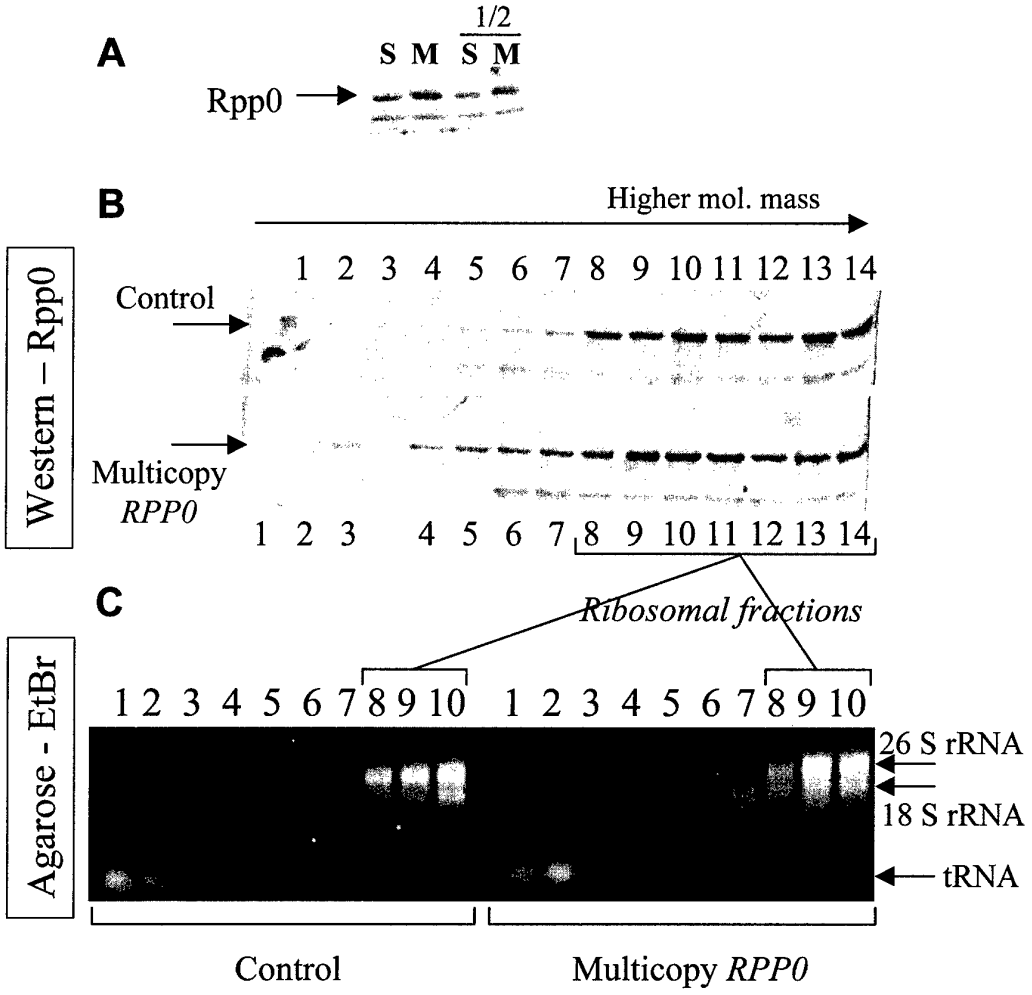

Figure 2. Overproduced Rpp0 accumulates in sub-ribosomal fractions. Lysates were prepared from the PS-5V-H19 [*psi*$^-$] transformants with either the Yeplac195 vector (C, control) or multicopy *RPP0* plasmid (M, m*RPP0*) (**A**) Comparison of the Rpp0 levels by Western blotting of total lysates; S, 50% load. (**B**) The lysates were fractionated by centrifugation and analyzed for Rpp0 by Western blotting. The Rpp0 levels are increased compared to control in sub-ribosomal fractions 3 to 7. (**C**) Determination of the fractions containing ribosomes. The same fractions were separated on agarose gel and stained with Ethidium Bromide. The 26S and 18S ribosomal RNAs are present in fractions 8 and higher.

many proteins, including chaperones. As a particular case of the indirect mechanism, the [PSI^{+}_{PS}] loss could be due to decreased expression of Sup35PS. Indeed, earlier we have observed the [PSI^{+}] loss at decreased Sup35 levels (unpublished data). However, here we found that the overexpression of Ssn8, Sfl1 and Rpp0 did not reduce the Sup35 levels (Table 2 and Western blotting data, not shown). Furthermore, the overexpression of Rpp0 increased Sup35 levels about 2-fold, which should counteract with, rather than promote, the prion curing.

TABLE 2. Alteration of the SFL1, SSN8 and RPP0 copy number influences activity of the chaperone promoters. Expression of lacZ under the control of indicated promoters was determined in the PS-5V-H19 [psi^{-}] cells with described alterations of the SFL1, SSN8 and RPP0 genes. The most significant differences with the control are given in bold. Control, transformants with the YEplac195 vector.

Strain with	Promoters				
	HSE	STRE	SSA4	HSP104	SUP35
Multicopy SFL1	**4.10 ± 0.17**	**7.18 ± 0.17**	**12.41 ± 1.31**	**43.1 ± 1.9**	8.07 ± 0.27
Deletion of SFL1	1.72 ± 0.15	5.23 ± 0.32	**13.13 ± 1.05**	21.0 ± 0.2	6.17 ± 0.08
Multicopy SSN8	1.52 ± 0.21	**3.22 ± 0.35**	**2.31 ± 0.61**	18.0 ± 1.1	6.49 ± 0.52
Deletion of SSN8	1.54 ± 0.11	**7.47 ± 0.24**	**12.72 ± 1.74**	26.6 ± 3.0	4.75 ± 0.36
Multicopy RPP0	**3.45 ± 0.21**	5.21 ± 0.44	**14.02 ± 2.53**	24.5 ± 3.1	**11.58 ± 0.77**
Control	1.41 ± 0.12	4.73 ± 0.51	5.16 ± 1.14	20.5 ± 0.5	6.77 ± 0.13

If Sfl1 and Ssn8 regulate expression of the chaperone genes, they would affect the levels of many chaperones and the curing of prions will likely be due to a combination of changes in the levels of several chaperones, rather than of any single chaperone. It is known that expression of the chaperone-encoding genes usually is controlled by either one or combination of the two promoter elements, HSE and STRE [27]. To characterize the effects of overproduced Sfl1, Ssn8, and Rpp0 on the chaperone expression, we determined their influence on the levels of lacZ expression under the control of model promoters containing either HSE or STRE. We also tested the activity of SSA4 and HSP104 promoters (Table2). SSA4 is an example of a gene expressed only under heat shock or stress conditions, while HSP104 is known for its unique and essential role in prion propagation. Excess Sfl1 increased the HSE-driven expression about 3-fold, which is consistent with its binding to the HSE element, and suggests that Sfl1 acts here as transcriptional activator, rather than repressor. Excess Sfl1 also increased the STRE-dependent expression. Excess Ssn8 did not affect the level of HSE-driven expression but repressed STRE promoter 1.5-fold and the SSA4 promoter 2-fold. The lack of Ssn8, conversely, activated the STRE- and SSA4-controlled expression, consistent with its repressor function. Excess Rpp0 increased the HSE- and SSA4-driven expression to approximately the same levels as excess Sfl1.

3.2. Mechanisms of action of the identified anti-prion factors

In this work, a search for the cellular factors interfering with the [PSI^{+}_{PS}] propagation was conducted. While previously only chaperones (Hsp104, Ssb1, Ssa1 and Ydj1) were known as such factors, this search revealed, in addition to chaperones Sis1, Apj1 and Sti1, transcriptional factors Sfl1 and Ssn8 and acidic ribosomal protein Rpp0. We propose that all revealed anti-prion factors could act either by directly interfering with the Sup35PS prion conversion, or by altering the levels of the proteins involved in this process. The former mechanism appears likely for the chaperones, while the latter for the transcriptional factors and the ribosomal protein.

Chaperones that cure yeast prions - Two of the three newly found chaperones, Sis1 and Apj1 (Ynl077w) belong to the Hsp40 family homologous to bacterial DnaJ chaperone. For Apj1, this is a first observation of a phenotypic effect, and it supports a chaperone function of this protein. Apj1 shows highest similarity to Ydj1 among numerous yeast DnaJ homologues.

No chaperones of the Hsp70 family were found in this screen. This is not surprising, since these chaperones are tightly autoregulated and allow a significantly lower increase of their expression than Hsp40 chaperones. For example,

Ssa1 had rather weak and Ssb1 had no effect on [PSI^{+}_{PS-1}] [21]. Another chaperone found in this screen is Sti1. This protein represents a component of the Hsp90-Hsp70 complex [29]. Thus, it is related to both Hsp90, which did not influence the [PSI^{+}] propagation, and Hsp70 (Ssa1 and Ssa2), which interfered with [PSI^{+}_{PS}], but reduced the [PSI^{+}] curing by excess Hsp104 [22]. Sti1 could interfere with prion formation either directly, or by modulating the activity of Ssa chaperones at the functional level. Also, the overexpression of Sti1 was shown to increase the Ssa4 expression [30], and thus it could act indirectly by altering the expression of Ssa or other chaperones.

Two kinds of anti-prion effects were expected in this work: the [PSI^{+}_{PS}] curing and the antisuppression due to the increased level of soluble functional Sup35PS. A significant antisuppression was only observed for the overexpressed Sis1. Earlier, the lack of antisuppressor effect of overexpressed Hsp104 against [PSI^{+}_{PS}] was noted, as a major difference from the case of [PSI^{+}]. However, in these cases a significant portion of both Sup35PS and Sup35 was found in soluble fractions of cell lysates [23]. It was proposed that in the [PSI^{+}_{PS}] case the apparently soluble Sup35PS obtained from the prion aggregates due to action of Hsp104, was not functionally reactivated, being in the form of small aggregates. The antisuppressor effect of Sis1 suggests that the excess Sis1 allowed to reactivate Sup35PS. The reactivation of aggregated proteins requires combined action of Hsp104, Ssa1 and Ydj1, as it was shown for denatured luciferase [26]. The same may apply for prion aggregates. Hsp104 is required for breaking large aggregates into small ones, from which Sup35PS may be refolded by Ssa1 and Ydj1. Since Sis1 is a homologue of Ydj1, it could substitute for Ydj1 in this mechanism. The described data support this and suggest that Sis1 is more efficient than Ydj1 in reactivation of prion Sup35PS. It should be noted that Ydj1 is a major cellular Hsp40, while Sis1 is usually expressed at 10-15-fold lower levels [31]. Thus, standard levels of Sis1 may be insufficient for the efficient reactivation of prion Sup35PS.

Non-chaperone proteins curing [PSI^{+}_{PS}] affect the expression of stress-inducible genes - One of these proteins, Sfl1, is a transcriptional factor structurally similar to the heat shock factor Hsf1, which plays a key role in the heat shock response. In contrast to Hsf1, the previously described effects of Sfl1 were mainly related to the cell wall biogenesis [32]. Furthermore, Sfl1 is presumed to mediate the transcriptional repression, rather than activation [33]. Sfl1 is highly similar to Hsf1 in the region corresponding to its DNA-binding domain, which suggests that Sfl1 could also bind to HSE and thus regulate chaperone expression. We confirmed this by finding that purified Sfl1 bind to HSE in vitro. Capacity of the cell lysates to bind HSE correlated with the Sfl1 levels and was rather low in the lysate lacking Sfl1. This suggests that Sfl1 was one of the major proteins bound to HSE under the given experimental conditions. The ability to bind to HSE was also shown for Skn7 [34], and could be expected of Mga1 and Hsm2 basing on sequence similarity. Apparently, these proteins and Sfl1 should compete with Hsf1 for binding to HSE, thus performing a complex regulation of HSE-dependent expression in response to different environmental challenges. Sfl1 did not bind to the STRE. Nevertheless, the amount of the STRE-protein complex in cell lysates notably depended on Sfl1, being increased both in lysate with excess Sfl1 and in lysate lacking it. This suggests that Sfl1 has a significant indirect influence on the STRE-controlled expression.

Another transcription factor revealed by [PSI^{+}_{PS}] curing is Ssn8, which represents a subunit of the transcriptional mediator complex [35]. Ssn8 represents a homologue of human cyclin C, and it degrades upon entry to meiosis and heat shock. Ssn8 acted as transcriptional repressor of SSA1 and presumably some other chaperone genes [36]. Importantly, Ssn8 was shown to interact with Sfl1 both physically and functionally. These proteins coimmunoprecipitated from cell lysates and both participated in the transcriptional repression of the SUC2 gene [33]. Since Sfl1 and Ssn8 are involved in transcription, their excess is likely to affect the expression of many proteins.

To characterize the influence of the excess Sfl1 and Ssn8 on the overall chaperone expression, we used model promoter constructs with the lacZ reporter gene. The excess of Sfl1 activated the HSE promoter 3-fold, which agrees with the Sfl1 binding to this element and suggests that Sfl1 acts as activator, rather than repressor for this element. It also induced the STRE-driven expression, but only 1.5-fold. This also agrees with the observed increase of the amount of protein bound to this DNA fragment in cell lysate with increased Sfl1 levels. In contrast, the excess of Ssn8 acted only on the STRE promoter and decreased its activity 1.5-fold, while the lack of Ssn8 induced it 1.5-fold. These data are consistent with the proposed Ssn8 function, as a repressor of transcription [37]. Thus, both Sfl1 and Ssn8 should affect significantly the expression of many chaperones, which is likely to be a cause for their prion-curing effects. It is noteworthy that in contrast to the earlier data, Sfl1 and Ssn8 exerted essentially different influence on transcription. This may indicate that these proteins do not always cooperate in their action.

Although the ribosomal protein Rpp0 is not supposed to participate in transcription, we observed that overproduced Rpp0 increased the HSE and SSA4-driven lacZ expression 2.5-fold, which could be sufficient for the observed [PSI^{+}_{PS}] curing. At the same time, the STRE and HSP104 promoters showed little activation. Since the translated message, lacZ, was the same, the difference in the effects of Rpp0 was defined at the transcriptional level. We suggest that the excess of Rpp0 may affect transcription either by directly participating in it, or indirectly. The latter opportunity is related to observation that the lack of acidic ribosomal proteins Rpp1A/B and Rpp2A/B, structurally

and functionally related to Rpp0, significantly affected the expression of many proteins and chaperones in particular [38]. It was proposed that the altered content of acidic proteins in ribosome changed its specificity towards different mRNAs. It appears unlikely that overproduction of Rpp0 would alter its content in ribosomes, since it is difficult to imagine that a ribosome can accommodate more than one molecule of this protein. However, the ribosome-free Rpp0 accumulated upon its overexpression may titrate a significant portion of Rpp1/2, thus creating a sub-population of ribosomes, which lack these proteins. This, in turn, may alter the synthesis of transcriptional factors that regulate the expression of chaperones.

3.3 New biochemical approach for studying the structural organization of the yeast prion particles

The data obtained in this work, as well as results of other studies showed that that different chaperones could influence the [PSI$^+$] propagation. However, the most remarkable effects were observed for Hsp104, which was the only chaperone strictly required for propagation of [PSI$^+$] and other prions. The overexpression of Hsp104 caused frequent [PSI$^+$] loss or antisuppressor effect when [PSI$^+$] was not lost [19]. Hsp104 has a unique role in the stress response. It is required to break large aggregates of denatured protein into smaller pieces, but it does not prevent aggregation or denaturation and cannot refold proteins to their native form [26, 39] . We proposed that Hsp104 acts similarly on prion aggregates of filamentous shape, causing their fragmentation into shorter filaments [17]. This should create new prion particles, which is essential for their inheritance, and accelerate the prion conversion by multiplying the ends of prion polymers, where the conversion occurs. The overproduction of Hsp104 should cause excessive fragmentation, increased levels of soluble Sup35 and possibly [PSI$^+$] loss. An alternative model proposed that Hsp104 is primarily required to facilitate the prion conversion in one or another way [12, 40].

The two models for the role of Hsp104 in prion replication may be distinguished, since they make different predictions for alteration of the size of prion particles upon inhibition of the Hsp104 function. By the former model, the size should increase due to blocked fragmentation, while by the latter it should stay constant or decrease due to block of polymerization. Recent studies provided some support for the "fragmentation" model. Decrease of the Hsp104 expression caused increase in the size of Sup35 prion aggregates, suggesting decreased disaggregation by Hsp104 [41]. The activity of Hsp104 may be inhibited by growing yeast cells in the presence of low concentrations of Guanidine-HCl (GuHCl) [42]. This treatment is known to cure efficiently [PSI$^+$] [43] and other known yeast prions. Study of the kinetics of [PSI$^+$] loss in the presence of GuHCl allowed to conclude that it blocks replication of prion "seeds" [44, 45]. Thus, inhibition of Hsp104 correlates with the block of fragmentation (replication) of prion particles (seeds). However, in these experiments the relation of the studied prion entities to the Sup35 polymers considered by the above models was not characterized. The prion seeds were defined genetically, but their physical nature was not studied. The size of prion aggregates was estimated by fluorescence of Sup35-GFP fusions or immunofluorescent staining [41]. However, this approach insufficiently characterizes the role of Hsp104 for a number of reasons: (i) the fluorescence is likely to show only largest aggregates, but not all of them; (ii) it is not clear, whether the observed fluorescent foci represent single prion particles or their higher-order complexes and (iii) the lack of quantitative estimates does not allow to conclude whether Hsp104 is critical for the fragmentation or just moderately modulates it.

To study the structure of prion aggregates and characterize the role of Hsp104, we developed here a novel approach for purification of prion particles and determination of their size. Using it, we showed that the Sup35 prion aggregates observed in vivo represent agglomerations of relatively small prion core polymers with amyloid-like properties. The size of these polymers varied significantly between different [PSI$^+$] variants. It depended greatly on the activity of Hsp104 in a manner, which strongly supports the idea that Hsp104 mediates fragmentation of Sup35 prion polymers.

Native [PSI$^+$] prion particles contain multiple Sup35 prion polymers - To check the available models for the structure and maintenance of yeast prions, it was required to determine the size of yeast prion particles. However, the methods, which would allow doing this with a reasonable precision, were unavailable. This prompted us to develop an electrophoresis-based approach for such analysis. Since it may be presumed that the prion particles may be associated with other molecules, macromolecular complexes and with each other, we had first to find the conditions, which destroy associations of prion particles with other molecules and with each other, but do not disrupt these particles. Earlier, GuHCl at high concentrations was used to obtain Sup35 prion aggregates containing no detectable contaminants [11]. However, we found later that while GuHCl solubilized the majority of cellular proteins, it also caused secondary precipitation of some proteins of yeast lysates (data not shown). We tested several other reagents at different concentrations for their ability to purify prion particles, including urea, high salt (KCl), deoxycholate, zwittergent 3-10, cetyl-trimethyl ammonium bromide, N-lauroyl sarcosyl and sodium dodecyl sulfate (SDS). For this, the aggregated fraction of 5V-H19 [PSI$^+$] lysates was dissolved in buffers containing these reagents, centrifuged at high speed and the proportion of Sup35 to other proteins in the pellets was studied (data not shown). The best

purification of Sup35 was achieved with N-lauroyl sarcosyl and SDS applied at room temperature. Further tests were focused on the use of SDS, which had minor advantages over sarcosyl.

It was important to confirm that the presence of SDS does not impair the Sup35 prion particles. For this, the aggregate fraction of 5V-H19 [PSI$^+$] lysate was dissolved in the Laemmli sample buffer containing 2% SDS and aliquots were incubated at different temperatures ranging from 20 to 100°C. Without further boiling the samples were subjected to SDS-PAGE and analyzed for Sup35 by Western blotting (Fig.3A). Sup35 was represented by two bands: a monomer band of about 80 kDa and polymers stuck at the top of the separating gel. In the samples incubated at 42°C or lower temperatures, low levels of monomers were observed, which are likely to represent ribosome-bound monomeric Sup35. Thus, Sup35 prion particles were resistant to SDS at these temperatures. They dissolved slightly at 50°C, and almost completely at 70°C and higher temperatures. Earlier, a similar ability to withstand SDS at room temperature was demonstrated for Sup35 amyloid-like polymers obtained in vitro [6]. This supports the structural similarity of Sup35 prion particles and Sup35 amyloid fibers.

The described properties of SDS allow its use for electrophoretic analysis of the prion particles. However, since boiling of the samples was excluded, correct estimation of the mass of Sup35 prion particles required showing that no additional proteins remained associated with them in the presence of SDS. For this, we analyzed the proteins trapped at the top of the separating gel together with prion Sup35. A strip of the separating gel, containing Sup35 oligomers, was cut off, boiled in SDS sample buffer and the proteins separated using another SDS-PAGE gel as described in Fig. 3B. In this way, one can observe the proteins, which run as aggregates before boiling, but as monomers after boiling. These should include Sup35 prion, other proteins with similar properties and the proteins associated with Sup35 particles during the first electrophoresis. The only abundant protein band represented Sup35 (Fig. 3B), as confirmed by immunoblotting (not shown). Although in this experiment some minor proteins could escape detection by Coomassie Blue, it is possible to conclude that SDS dissolves most of the yeast non-prion protein complexes and removes most of the proteins, which could be associated with Sup35 prion core particles.

The large size of prion particles, presumably of the megaDalton range, precluded the use of polyacrylamide gels, even at lowest possible concentration of about 3%. So, in further experiments agarose gels were used. As a molecular mass standard, the preparation of rabbit myofibrils, which contains giant proteins titin and nebulin, was used [46]. We assumed that the molecular masses of titin and nebulin are equal to 4200 kDa and 740 kDa, which corresponds to the coding capacities of respective human genes [47, 48]. The homologous rabbit genes were not completely sequenced, but the partial amino acid sequence of rabbit titin was highly similar (99%) to its human counterpart [47].

A range of SDS concentrations for the treatment of samples was tried using 5V-H19 [PSI$^+$] lysate, to check that they do not affect the size of prion particles. The samples were analyzed using horizontal agarose gel as described in Experimental procedures (data not shown). The mobility of Sup35 oligomers did not increase, when the SDS concentration increased from 0,5 to 5% or duration of treatment increased from 3 to 10 min. This confirms that SDS does not impair Sup35 polymers: otherwise, we would observe a decrease in the polymer size with the increase of SDS concentration and duration of treatment. The polymer mobility was somewhat decreased at SDS concentrations lower that 0,5%. Most likely, these low levels of SDS were insufficient to destroy all non-prion interactions. In further work, buffers containing 2% SDS were used for incubation of lysate samples at 37°C. This removes the associated proteins from Sup35 prion polymers, but leaves them intact and allows to analyze their size using agarose gel containing 0.1% SDS. This method was called Semi-Denaturing Detergent - Agarose Gel Electrophoresis (SDD-AGE).

A comparison of [PSI$^+$] and [psi$^-$] lysates with this method distinguished them clearly (Fig. 4). In [PSI$^+$] lysate, Sup35 was mostly in the polymer form, while in [psi$^-$] it was monomeric. In contrast, in the centrifugation analysis a significant portion of Sup35 was usually found in the aggregated fraction of both [PSI$^+$] and [psi$^-$] lysates (Fig. 5A). The prion polymers were heterogeneous in size, ranging from 700 to 4000 kDa, which should correspond to 9 to 50 Sup35 monomers. This approach also allowed to observe polymers of another prion protein, Rnq1, in [PIN$^+$] cells and the lack of such polymers in [pin$^-$] cells (data not shown).

Since we found that SDS removes associated proteins from Sup35 prion polymers, it was of interest, how the size of such "core" particles compares to the size of Sup35-containing aggregates observed under the native conditions used in standard centrifugation methods. For this, we analyzed the lysate of 5V-H19 [PSI$^+$] strain by centrifugation in the presence of 2% SDS and its absence. Comparison of the Sup35 sedimentation profiles showed that the presence of SDS greatly reduced the size of Sup35 prion aggregates (Fig. 5A). On average, the mass of Sup35 prion aggregates was 35-fold greater than the mass of prion cores (see Experimental Procedures). So great difference in sizes of prion polymers and aggregates may be due to two reasons, the association of Sup35 polymers in aggregates with some other proteins and macromolecular complexes, such as ribosome, and the presence of more than one Sup35 polymer per

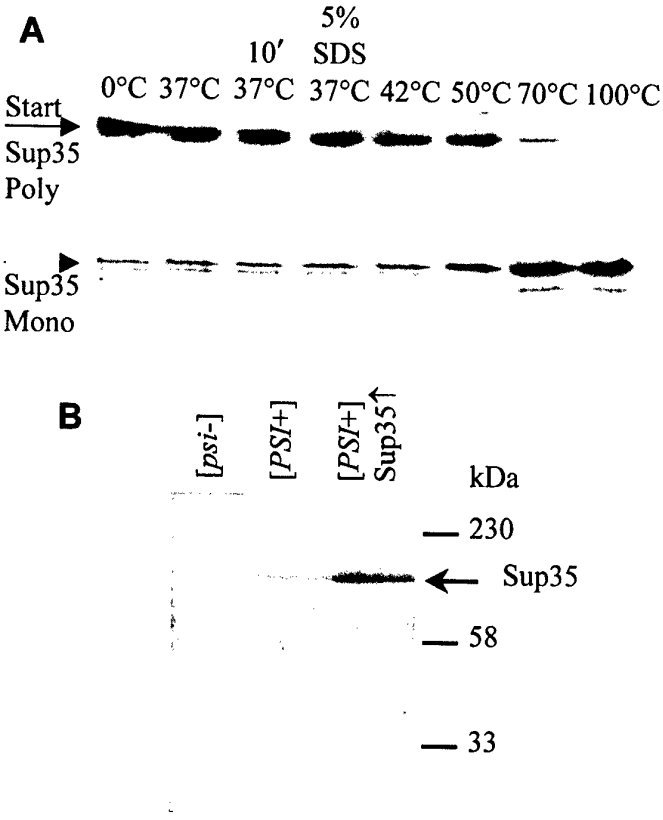

Figure 3. Sup35 prion particles are the only protein macromolecular complexes resistant to SDS at low temperature.
(A) The aggregated fraction of 5V-H19 [*PSI*$^+$] lysate was incubated in the loading buffer containing 2% SDS, or 5%, where shown, at indicated temperatures for 3 min or, in one case, for 10 min and analyzed by SDS-PAGE. Arrows indicate Sup35 monomers and polymers trapped at the top of gel. Immunostaining for Sup35.
(B) Aggregate fractions of the lysates of 5V-H19 [*psi*$^-$], [*PSI*$^+$] cells and [*PSI*$^+$] cells overexpressing Sup35 were mixed with SDS sample buffer, incubated for 5 min at 37°C and separated by SDS-PAGE. The stacking gel was made of 1,8% agarose instead of polyacrylamide to alleviate the passage of large particles. The top 3 mm of separating gel was cut out, boiled in a sample buffer containing 2% SDS and placed on the top of another SDS-PAGE gel. After electrophoresis, the gel was stained with Coomassie Blue. The identity of the Sup35 band was confirmed by immunoblotting (not shown).

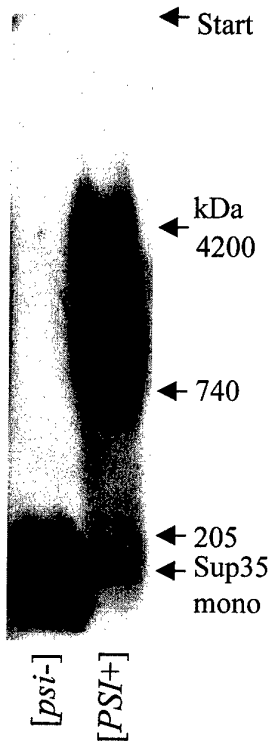

Figure 4. Comparison of [*PSI*$^+$] and [*psi*$^-$] variants of 5V-H19 by SDD-AGE with immunoblotting for Sup35. Myo, rabbit myofibrillar proteins used for molecular mass standards: titin, 4200 kDa, nebulin, 740 kDa and myosin heavy chain, 205 kDa; blot staining with Ponceau S. A band of about 3000 kDa probably represents a shorter expression variant of titin.

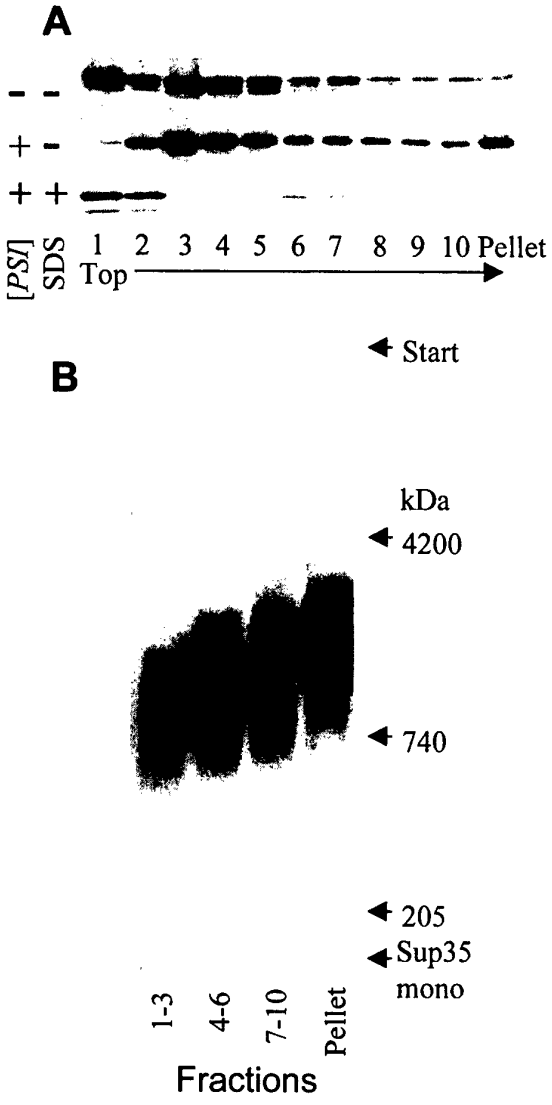

Figure 5. SDS disassembles Sup35 prion aggregates into smaller SDS-resistant particles.

(A) The lysates of 5V-H19 [*psi*⁻] and [*PSI*⁺] strains were fractionated by centrifugation at 100 000g for 15 min in the absence (-) or presence (+) of SDS. The fractions were analyzed by SDS-PAGE.

(B) The fractions of [*PSI*⁺] lysate centrifuged without SDS were pooled as indicated and analyzed by SDD-AGE. Molecular mass standards are indicated. Immunostaining for Sup35.

aggregate. However, the relative contribution of these two factors is difficult to determine. The Sup35 prion aggregates varied greatly in their size. The smallest of them were comparable to prion cores, the largest were about 100-fold bigger. However, the prion cores, constituting these aggregates differed much less in their size. The centrifugation fractions obtained without SDS were analyzed by SDD-AGE (Fig. 5B). The average size of Sup35 polymers in the slowest sedimenting fraction was about 900 kDa, and in the pellet about 1500 kDa, which constitutes only 1,7-fold difference.

[PSI$^+$] variants differ in the size of Sup35 polymers - Different isolates of [PSI$^+$] may vary in the strength of the nonsense suppressor phenotype and in mitotic stability [49]. Such variation was also observed for hybrid prion [PSI$^+_{PS}$] based on Sup35 prion domain from yeast Pichia methanolica [23]. Usually, stronger suppression correlates with higher stability. It was proposed that the variation in [PSI$^+$] properties reflects the difference in structure of prion particles [21, 50, 51]. This may result in variation of prion polymerization speed and the frequency of fragmentation of prion polymers, and, therefore, in their different size [17]. To check this, we compared the size of Sup35 polymers in different [PSI$^+$] isolates. A significant variation was found, with the size being inversely related to the strength of [PSI$^+$] (Fig. 6). Strong [PSI$^+$] isolates generally had smaller Sup35 polymers than weak ones. Therefore, the cells harboring strong [PSI$^+$] should have more prion polymers, which agrees with higher mitotic stability of such [PSI$^+$] and more efficient prion formation, reflected by lower levels of soluble functional Sup35. Among hybrid [PSI$^+_{PS}$], strong variants also had smaller Sup35 polymers, though with one exception: the strong [PSI$^+_{PS\text{-}1}$] showed large Sup35 polymers. The minimal size of prion polymer observed among the studied [PSI$^+$] variants corresponded to approximately 600 kDa, or about 8 Sup35 monomers.

GuHCl inhibits fragmentation of Sup35 prion polymers - GuHCl was shown to inactivate Hsp104, which should be a reason of its prion curing effect [42, 45]. To investigate the effect of GuHCl at the level of prion cores, the 5V-H19 [PSI$^+$] strain was grown in the YPD medium containing 3 mM GuHCl. Half volume of the culture was taken for analysis once per generation and replaced with an equal amount of fresh medium with GuHCl to keep the culture density constant. After the third generation, the cells were transferred to YPD lacking GuHCl and aliquots were taken once per hour. Cell lysates were obtained and analyzed by SDD-AGE (Fig. 7A). In the presence of GuHCl the average size of the Sup35 polymers increased about 2-fold per generation. Therefore, the fragmentation of Sup35 cores was impaired, rather than their growth by accretion of new Sup35 molecules. Furthermore, the observed polymer growth rate represents a maximum, achievable on conditions that all Sup35 monomers incorporated into polymers and that the polymers did not experience fragmentation. Indeed, in such case the total number of Sup35 prion polymers remains constant, but at each generation the number of cells doubles and therefore the number of Sup35 polymers per cell should decrease twofold. Since the amount of Sup35 per cell remains constant and most of it is in the polymer form, the number of Sup35 molecules per polymer should double. This allows concluding that GuHCl entirely blocks the fragmentation of Sup35 prion polymers. The doubling of polymer size at the first generation suggests that the fragmentation was blocked immediately after addition of GuHCl. After removal of GuHCl from the medium, the size of Sup35 polymers was unchanged for about 1 hour and then gradually returned to the starting values. The delay in decrease of the size of prion cores could reflect the time required for washing out of GuHCl from the yeast cells.

Reduced levels of Hsp104 increase the size of Sup35 prion polymers - To study the effects of the lack of Hsp104 directly, the chromosomal HSP104 gene was put under control of the tetracycline-regulatable TET promoter. This allowed to inhibit the synthesis of Hsp104 by addition of antibiotic to culture medium. However, it was not possible to achieve a rapid decrease of the Hsp104 levels, since this chaperone degrades very slowly and its amount should decrease mainly due to dilution at cell divisions [41]. In the absence of antibiotic, the TET promoter was induced and provided about twofold increased level of Hsp104 compared to the native HSP104 promoter. This difference did not alter significantly the size of Sup35 polymers and did not cause antisuppression or [PSI$^+$] loss, often associated with excess Hsp104. The experiment was started with addition of the tetracycline analog, doxycycline, to 5μg/ml to repress the TET promoter. Culture aliquots were taken once per generation and cell lysates were analyzed by SDD-AGE. The size of Sup35 polymers started to increase after 2 generations of HSP104 repression, reaching about four-fold increase after 6 generations (Fig. 7B). This is a significantly slower increase than the one caused by GuHCl, which should be related to the presence of decreasing amounts of Hsp104 during the experiment. The basal level of Hsp104, determined after 2 days of growth in the presence of doxycycline, was about 5% of its wild type levels.

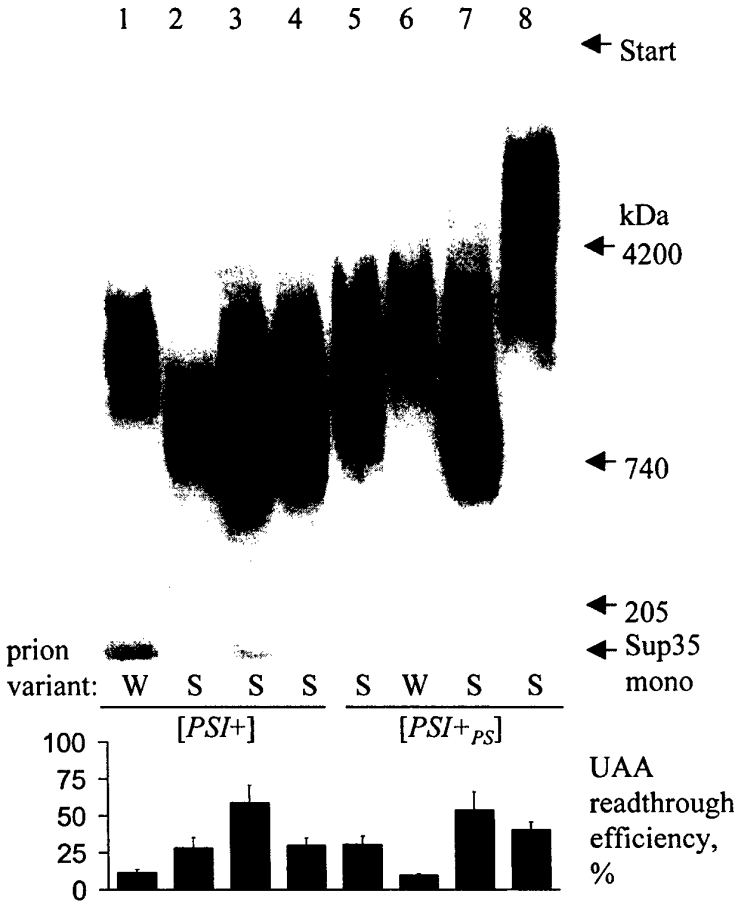

Figure 6. Comparison of the size of Sup35 prion polymers by SDD-AGE and of the efficiency of UAA nonsense codon readthrough in different [*PSI*$^+$] and [*PSI*$^+_{PS}$] variants of the 5V-H19 and PS-5V-H19 strains. Prion variants: W, weak; S, strong. Lanes: 1, [*PSI*$^+_6$]; 2, [*PSI*$^+_1$]; 3, [*PSI*$^+_{11}$]; 4, [*PSI*$^+_{37}$]; 5; [*PSI*$^+_{PS\text{-}20}$]; 6, [*PSI*$^+_{PS\text{-}7}$]; 7, [*PSI*$^+_{PS\text{-}2}$]; 8, [*PSI*$^+_{PS\text{-}1}$]. Molecular mass standards are indicated. Immunostaining for Sup35.

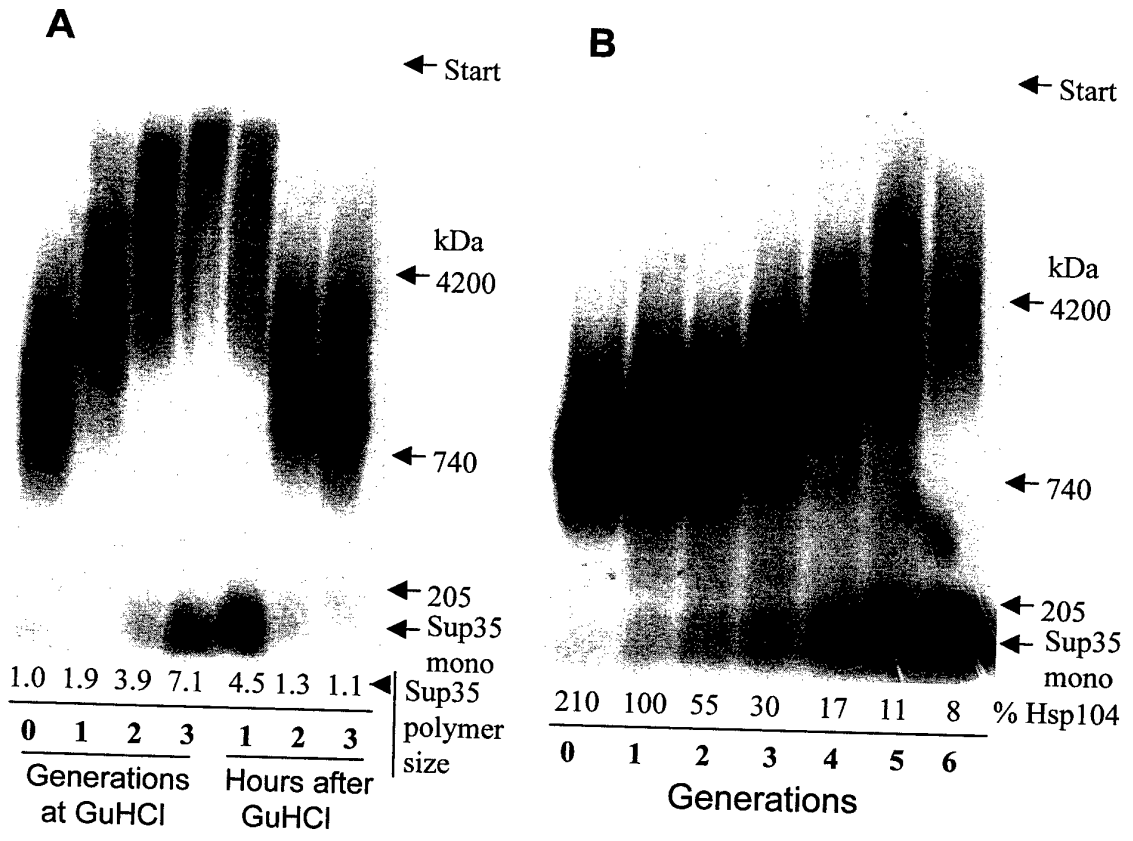

Figure 7. The effects of GuHCl (A) and decreased (B) expression of Hsp104 on the size of Sup35 prion polymers. SDD-AGE analysis with immunostaining for Sup35.

(A) The 5V-H19 [*PSI*$^+$] cells were grown for 3 generations in the presence of 3mM GuHCl and then for 3 hours in the absence of GuHCl. The average size of Sup35 polymers relatively to the wild type is indicated.

(B) The expression of Hsp104 in the strain T104-5V-H19 was repressed for 6 generations. The levels of Hsp104, determined by scanning SDS-PAGE blots of the samples stained for Hsp104, are indicated as % relatively to the wild type level.

3.4. Two-level structural organization of the yeast prion aggregates and a role of the Hsp104 chaperone in the [PSI$^+$] prion propagation

Polymers and aggregates: two levels in the structure of yeast prions - Yeast prions, the same as prions of higher eukaryotes, are presumed to be structurally similar to amyloid fibers [52]. This conclusion is related in part to observations that the yeast prionogenic proteins Sup35 and Ure2 tend to form amyloid fibers in vitro [14, 15]. In [PSI$^+$] cells, yeast prions form large aggregates [11]. However, no direct data were available on the structure and composition of these aggregates and their relation to amyloid fibers. Here, we showed that these aggregates represent agglomerations of a number of relatively small Sup35 prion core polymers and other associated proteins. The prion polymers were physically different from all other protein complexes: while the majority, if not all, of protein complexes of yeast lysates were dissolved in 2% SDS at 37°C, the prion cores were resistant to this condition. This unique property of prion cores is also typical of the Sup35 amyloid fibers formed in vitro [6]. This strongly suggests that the prion cores represent amyloid fibers. It is also interesting that while non-prion protein complexes were dissolved by SDS, many individual proteins retain their function and therefore structure in its presence. For example, 1% SDS did not affect the activity of Proteinase K [53], yeast cellular proteases and the fluorescence of Green Fluorescent protein (data not shown). Thus, the interactions between monomers within the prion polymer are comparable by their strength to intraprotein interactions.

By the amyloid model [5], a certain minimal number of monomers is required for a stable amyloid polymer. This number is related to the number of monomers per turn of helical structure of polymer and may define the kinetics of appearance of amyloid seeds de novo. The smallest polymers observed in our work contained about 8 monomers. However, we do not exclude that smaller polymers could be stable, but in vivo they were preferentially eliminated by chaperones.

By centrifugation, the Sup35 prion cores were on average about 35-fold smaller than the aggregates, which they form. SDD-AGE revealed that the cores comprised on average about 15-30 Sup35 molecules, depending on the prion variant. This value may be used to estimate the cellular number of prion cores. It is possible to suggest that there are about 30,000 Sup35 molecules per yeast cell. Although this value was not directly determined for Sup35, it was obtained for Sup45 [54], a functional partner of Sup35. These proteins form a heterodimer functioning in the translation termination [9], their genes show similar codon bias indexes and the SUP35 mRNA is slightly more abundant than the SUP45 one [54]. This allows suggesting that Sup35 and Sup45 are expressed at similar levels. With an average prion polymer containing 20 Sup35 molecules, there should be about 1500 such polymers per cell. On the other hand, it was calculated that there are about 60 heritable prion units (seeds) per cell [44]. These heritable units are likely to be equivalent to the Sup35 aggregates. Then there could be about 25 prion polymers on average per heritable prion aggregate, which agrees well with our centrifugation data.

It is interesting to note that the prion polymers and aggregates are relatively independent in defining the prion phenotypes. The prion conversion and the suppressor phenotype should depend on the number and properties of the polymers, while the stability of inheritance depends on the number of aggregates. These numbers usually correlate, but at least in one case, that of Hsp104 overexpression, the correlation was broken: the number of polymers decreased, while the number of aggregates increased. The ability of prion polymers to catalyze further polymerization implies that a polymer represents a minimal particle able to act as a heritable seed. The aggregation of several polymers into a single particle reduces their seeding potential, making them a single seed, but this should not impair the efficiency of polymerization. It should be noted that the term "prion aggregates" does not necessarily imply the presence of multiple polymers in them. This term was only used to denote the prion-containing particles revealed by centrifugation in the absence of SDS. The smallest aggregates were almost of the same size as prion cores and thus were likely to contain just one prion core.

The prion visualization using Sup35-GFP fusions usually reveals most of prion Sup35 being localized in several bright foci [12], which contrasts with the several tens of prion seeds calculated earlier [44]. Apparently, these foci represent the largest prion aggregates, which, though comprising most of cellular Sup35, constitute only a small portion of prion seeds. The majority of seeds should be too small to be detectable by fluorescent microscopy. Thus, despite the convenience of GFP test for prion detection, it may not be used for judging quantitative aspects of prion aggregation. Also, in some cases no foci were detected in the cells containing prion-GFP fusions [55, 56]. This phenotype is compatible with prions being polymers, but with a low degree of aggregation.

[PSI$^+$] variants differ in properties of Sup35 prion polymers - Earlier it was proposed that the variation of the [PSI$^+$] properties, known as "prion strains" or "prion variants" results from variation in the structure of prion particles [21, 50, 51]. In the frame of polymerization-fragmentation model, this means that the prions may differ in the polymerization speed (hereafter, the "speed" is used to characterize growth of a polymer; the "rate" describes overall

prion protein polymerization and equals to the product of the speed and the number of polymers in a cell) and in the efficiency of prion recognition and fragmentation performed by Hsp104 [17]. These parameters define the size of Sup35 polymers, which, therefore, may differ between prion variants. Indeed, we observed a significant variation in the length of prion polymers in independent isolates of both conventional [PSI^+] and hybrid [PSI^+_{PS}]. The length of polymers correlated inversely with the suppressor strength of the [PSI^+] variants, with a single exception, that of [$PSI^+_{PS\text{-}1}$]. Such correlation may be anticipated, as well as some exceptions from it. Smaller polymers mean more prion particles per cell, higher polymerization rate, lower levels of monomeric Sup35 and higher nonsense suppression. In this logical sequence, however, one step is not strict: the polymerization rate is proportional to the number of prion particles, but it is also proportional to the polymerization speed. The correlation observed among [PSI^+] variants suggests the lack of significant variation of this speed, but this may not be the case for some other [PSI^+] variants. A further reservation should be made for the cells carrying strong [PSI^+_{PS}] variants, which contained very low levels of soluble Sup35. These levels were lower than those in [PSI^+] variants of similar suppressor efficiency and were below the 5-10% threshold, minimally required for the cell viability [57]. This forces to suggest that polymerized Sup35 retains, though decreased, functional activity. It appears likely that in the prion aggregates the functional C-terminal domain of Sup35 is not structurally rearranged, but its linkage to a bulky aggregate sterically interferes with its interaction with the ribosome and/or other essential targets. The steric interference could be decreased for hybrid Sup35PS, since its middle domain, which could act as a spacer between the prion and functional domains, is larger than in S. cerevisiae Sup35 (162 amino acids versus 130). Therefore, in the prion variants with very low levels of monomeric Sup35 the efficiency of translational suppression may depend on the structure of prion particles, while in other variants the suppression should primarily depend on the levels of soluble Sup35.

The increased Sup35 polymer length in weak prion variants suggests fewer prion core particles per cell, which agrees well with low mitotic stability of these variants. Although one can presume that prion stability depends on the number of prion aggregates, rather than core particles, these numbers should correlate, since longer polymers seem to have higher propensity to aggregate. Increased size of core particles should cause an even greater increase in the size of prion aggregates.

Hsp104 is required to fragment prion polymers - Hsp104 plays a key role in propagation of yeast prions, since it is the only chaperone, which is indispensable for their propagation. To explain the requirement for Hsp104 in prion propagation, we proposed that Hsp104 is necessary to fragment prion polymers [17], which is required for their inheritance and for faster prion conversion. An alternative model proposed that Hsp104 is required for the prion conversion coupled with polymerization by helping to obtain certain unfolded intermediate form of prionogenic protein [6, 12] . It is possible to discriminate between these models, since they make different predictions for the case of lack of Hsp104 function: the size of prion polymers should increase by the former model and should not by the latter. Here, the Hsp104 function was decreased in two ways: by decreasing its levels and by GuHCl mediated inhibition. In both cases a rapid increase in the size of Sup35 polymers was observed, which indicates a defect of fragmentation, rather than of polymerization. This supports the first model and contradicts to the second. Furthermore, the GuHCl experiment indicated a complete block of prion fragmentation. The size of Sup35 polymers grew twofold per cell generation, a rate that may only be achieved upon full block of fragmentation. It may be assumed that Hsp104 was solely responsible for the block, even though some other proteins involved in [PSI^+] propagation may also be affected by GuHCl. Since the [PSI^+] curing by GuHCl could be prevented by mutations in the HSP104 gene [45], other proteins are not critical for the fragmentation block. The levels of free monomeric Sup35 were not increased during the first generation in the presence of GuHCl, which also confirms that Sup35 prion polymerization was not inhibited.

It should be noted that Hsp104 functions both at the level of prion polymers and aggregates. Earlier data showed that Hsp104 acts to dissolve the aggregates [23, 41]. Here, we showed that it is essential for fragmenting the prion polymers. The processes of disaggregation and fragmentation are likely to differ significantly, despite they may be driven by an essentially similar action of Hsp104. Prion polymers are regular, while aggregates are likely to be irregular structures. The polymers grow via accretion of monomers; the aggregates, in addition, can grow via joining of preexisting aggregates. Besides, the polymers are more solid, since the aggregates, but not polymers are dissolved in the presence of SDS. Due to these differences, the polymers and aggregates may interact differently with various chaperones. For example, overexpression of Hsp104 makes aggregates smaller, but may enlarge the polymers.

It is interesting to note that the speed of prion polymerization increased during the GuHCl experiment. Since during the first two generations in the presence of GuHCl relatively small amount of soluble Sup35 accumulated, the cellular rate of polymerization was approximately equal to the rate of Sup35 synthesis. After the two generations, the number of polymers per cell decreased 4-fold and, therefore, the speed of Sup35 polymerization increased 4-fold. This shows that the polymerization speed in strong [PSI^+] variants is substantially restricted by the availability of soluble Sup35.

4. CONCLUSIONS

The properties of yeast prions suggest that they may be used as a model for studying both prions and amyloids of higher eukaryotes [52]. This implies that the factors similar to those found here may be active against human and animal prion and amyloid diseases. This is supported by observation that overexpression of Hsp40 and Hsp70 chaperones in human cell lines interfered with aggregation of proteins with expanded polyglutamine tracts and amyloidogenic immunoglobulin light chains [58, 59]. In this work we confirmed our previous [21] observation that the efficiency of the prion curing factors varied greatly depending on the prion strain and the primary structure of prion domain. This suggests that the use of screening systems based on different prions may allow finding more factors involved in the prion-like processes. On the other hand, this should complicate the prediction of anti-prion activity of a particular factor against a given prion or amyloid. The non-chaperone factors similar to those discovered in this work could be no less active against prions and amyloids than individual chaperones, since they allow to activate several chaperones simultaneously.

To obtain a deeper insight into the mechanisms of propagation of yeast prions, a novel approach for purification and analysis of prion particles was developed. This approach allowed to show that the Sup35 prion aggregates observed in vivo represent agglomerations of relatively small prion core polymers with amyloid-like properties. In contrast to other protein complexes, but similarly to amyloid fibers, these polymers are SDS-insoluble. The size of polymers is characteristic of a given [PSI^+] variant, and differs between the variants. Blocked expression of Hsp104 chaperone causes gradual increase in the size of prion polymers while inactivation of Hsp104 by guanidine HCl completely stops their fragmentation, which indicates indispensability of Hsp104 for this process.

The data obtained in this work suggest a dual role of Hsp104 in prion propagation: dismantling of prion aggregates into smaller pieces and fragmentation of prion polymers constituting these aggregates. This may result in three types of events important for the prion propagation and inheritance. New prion seeds may be generated by separating one or several polymers from an aggregate or by cleaving off a fragment of one polymer. New polymers may appear without new seed generation when a polymer is cleaved, but remains attached to the aggregate.

While this work supported the amyloid-like nature of the yeast prions, the final proof of it could be the electron microscopic observation of purified core polymers. It would also be of interest to use the approaches described here to analyze other prion and amyloid proteins and especially the mammalian prion, PrP.

8. LITERATURE CITED

1. Horwich A. and Weissman J.S. (1997) Deadly conformations - protein misfolding in prion diseases. Cell, **89**, 449-510.
2. Prusiner S.B., Scott M.R., DeArmond S.J. and Cohen F.E. (1998) Prion protein biology. Cell, **93**, 337-348.
3. Tran P.B. and Miller R.J. (1999) Aggregates in neurodegenerative diseases: crowds and power? Trends Neurosci., **22**, 194-197.
4. Prusiner S.B. (1991) Molecular biology of prion diseases. Science, **252**, 1515-1522.
5. Jarrett J.T. and Lansbury P.T. (1993) Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? Cell, **73**, 1055-1058.
6. Serio T.R., Cashikar A.G., Kowal A.S., Sawicki G.J., Moslehi J.J., Serpell L., Arnsdorf M.F and Lindquist S.L. (2000) Nucleated conformational conversion and the replication of conformational information by a prion determinant. Science, **289**, 1317-1321.
7. Wickner R.B. (1994) [URE3] as an altered Ure2 protein: evidence for prion analogue in Saccharomyces cerevisiae. Science, **264**, 532-535.
8. Coustou V., Deleu C., Saupe S. and Begueret J. (1997) The protein product of the het-s heterokaryon incompatibility gene of the fungus Podospora anserina behaves as a prion analog. Proc. Natl. Acad. Sci. USA, **94**, 9773-9778.
9. Stansfield, I., Jones, K.M., Kushnirov, V.V., Dagkesamanskaya, A.R., Poznyakovski, A.I., Paushkin, S.V., Nierras, C.R., Cox, B.S., Ter-Avanesyan, M.D., and Tuite, M.F. (1995) The products of the SUP45 (eRF1) and SUP35 genes interact to mediate translation termination in Saccharomyces cerevisiae. EMBO J., **14**, 4365-4373.
10. Zhouravleva, G., Frolova, L., Le Goff, X., Le Guellec, R., Inge-Vechtomov, S.G., Kisselev, L.L., and Philippe, M. (1995) Termination of translation in eukaryotes is governed by two interacting polypeptide chain release factors, eRF1 and eRF3. EMBO J., **14**, 4065-4072.

11. Paushkin S.V., Kushnirov V.V., Smirnov V.N. and Ter-Avanesyan M.D. (1996) Propagation of the yeast prion-like [PSI$^+$] determinant is mediated by oligomerization of the SUP35-encoded polypeptide chain release factor. EMBO J., **15**, 3127-3134.
12. Patino M.M., Liu J.J., Glover J.R. and Lindquist S. (1996) Support for the prion hypothesis for inheritance of a phenotypic trait in yeast. Science, 273, 622-626.
13. Paushkin S.V., Kushnirov V.V., Smirnov V.N. and Ter-Avanesyan M.D. (1997) In vitro propagation of the prion-like state of the yeast Sup35 protein. Science, **277**, 381-383.
14. Glover J.R., Kowal A.S., Schirmer E.C., Patino M.M., Liu J.-J. and Lindquist S.L. (1997) Self-seeded fibers formed by Sup35 the protein determinant of [PSI$^+$], a heritable prion-like factor of S. cerevisiae. Cell, **89**, 811-819.
15. King C.-Y., Tittmann P., Gross H., Gebert R., Aebi M. and Wuthrich K. (1997) Prion-inducing domain 2-114 of yeast Sup35 protein transforms in vitro into amyloid-like filaments. Proc. Natl. Acad. Sci. USA, **94**, 6618-6622.
16. Harper J.D. and Lansbury P.T., Jr. (1997) Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins. Annu. Rev. Biochem., **66**, 385-407.
17. Kushnirov V.V. and Ter-Avanesyan M.D. (1998) Structure and replication of yeast prions. Cell, **94**, 13-16.
18. Sparrer H.E., Santoso A., Szoka F.C. and Weissman J.S. (2000) Evidence for the prion hypothesis: induction of the yeast [PSI$^+$] factor by in vitro-converted Sup35 protein. Science, **289**, 595-599.
19. Chernoff Y.O., Lindquist S.L., Ono B., Inge-Vechtomov S.G. and Liebman S.W. (1995) Role of the chaperone protein Hsp104 in propagation of the yeast prion- like factor [PSI$^+$]. Science, **268**, 880-884.
20. Chacinska A., Szczesniak B., Kochneva-Pervukhova N.V., Kushnirov V.V., Ter Avanesyan M.D. and Boguta M. (2001) Ssb1 chaperone is a [PSI$^+$] prion-curing factor. Curr. Genet., **39**, 62-67.
21. Kushnirov V.V., Kryndushkin D.S., Boguta M., Smirnov V.N. and Ter Avanesyan M.D. (2000) Chaperones that cure yeast artificial [PSI$^+$] and their prion-specific effects. Curr. Biol., **10**, 1443-1446.
22. Newnam G.P., Wegrzyn R.D., Lindquist S.L. and Chernoff Y.O. (1999) Antagonistic interactions between yeast chaperones Hsp104 and Hsp70 in prion curing. Mol. Cell Biol., **19**, 1325-1333.
23. Kushnirov V.V., Kochneva-Pervukhova N.V., Chechenova M.B., Frolova N.S. and Ter Avanesyan M.D. (2000) Prion properties of the Sup35 protein of yeast Pichia methanolica. EMBO J., **19**, 324-331.
24. Sondheimer N., Lopez N., Craig E.A. and Lindquist S. (2001) The role of Sis1 in the maintenance of the [RNQ$^+$] prion. EMBO J., **20**, 2435-2442.
25. Lu Z. and Cyr D.M. (1998) Protein folding activity of Hsp70 is modified differentially by the hsp40 co-chaperones Sis1 and Ydj1. J. Biol. Chem., **273**, 27824-27830.
26. Glover J.R. and Lindquist S. (1998) Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell, **94**, 73-82.
27. Piper P. (1997) The yeast heat shock response. In Hohmann S. and Mager W.H. (Eds.), Yeast stress responses, R.G. Landes Company, New York, pp. 75-100.
28. Santos C. and Ballesta J.P. (1994) Ribosomal protein P0, contrary to phosphoproteins P1 and P2, is required for ribosome activity and Saccharomyces cerevisiae viability. J. Biol. Chem., **269**, 15689-15696.
29. Chang H.C. and Lindquist S. (1994). Conservation of Hsp90 macromolecular complexes in Saccharomyces cerevisiae. J. Biol. Chem., **269**, 24983-24988.
30. Nicolet C.M. and Craig E.A. (1989) Isolation and characterization of STI1, a stress-inducible gene from Saccharomyces cerevisiae. Mol. Cell Biol., **9**, 3638-3646.
31. Yan W. and Craig E.A. (1999) The glycine-phenylalanine-rich region determines the specificity of the yeast Hsp40 Sis1. Mol. Cell Biol., **19**, 7751-7758.
32. Fujita A., Kikuchi Y., Kuhara S., Misumi Y., Matsumoto S. and Kobayashi H. (1989) Domains of the SFL1 protein of yeasts are homologous to Myc oncoproteins or yeast heat-shock transcription factor. Gene, **85**, 321-328.
33. Song W. and Carlson M. (1998) Srb/mediator proteins interact functionally and physically with transcriptional repressor Sfl1. EMBO J., **17**, 5757-5765.
34. Raitt D.C., Johnson A.L., Erkine A.M., Makino K., Morgan B., Gross D.S. and Johnston L.H. (2000) The Skn7 response regulator of Saccharomyces cerevisiae interacts with Hsf1 in vivo and is required for the induction of heat shock genes by oxidative stress. Mol. Biol. Cell, **11**, 2335-2347.
35. Li Y., Bjorklund S., Jiang Y.W., Kim Y.J., Lane W.S., Stillman D.J. and Kornberg R.D. (1995) Yeast global transcriptional regulators Sin4 and Rgr1 are components of mediator complex/RNA polymerase II holoenzyme. Proc. Natl. Acad. Sci. U. S. A, **92**, 10864-10868.
36. Cooper K.F., Mallory M.J., Smith J.B. and Strich R. (1997) Stress and developmental regulation of the yeast C-type cyclin Ume3p (Srb11p/Ssn8p). EMBO J., **16**, 4665-4675.

37. Cooper K.F. and Strich R. (1999) Functional analysis of the Ume3p/ Srb11p-RNA polymerase II holoenzyme interaction. Gene Expr., **8,** 43-57.
38. Remacha M., Jimenez-Diaz A., Bermejo B., Rodriguez-Gabriel M.A., Guarinos E., and Ballesta J.P. (1995). Ribosomal acidic phosphoproteins P1 and P2 are not required for cell viability but regulate the pattern of protein expression in Saccharomyces cerevisiae. Mol. Cell Biol., **15,** 4754-4762.
39. Parsell D.A., Kowal A.S., Singer M.A. and Lindquist S. (1994) Protein disaggregation mediated by heat-shock protein Hsp104. Nature, **372,** 475-478.
40. Serio T.R. and Lindquist S.L. (2000) Protein-only inheritance in yeast: something to get [PSI^+]-ched about. Trends Cell Biol., **10,** 98-105.
41. Wegrzyn R.D., Bapat K., Newnam G.P., Zink A.D. and Chernoff Y.O. (2001) Mechanism of prion loss after Hsp104 inactivation in yeast. Mol. Cell Biol., **21,** 4656-4669.
42. Ferreira P.C., Ness F., Edwards S.R., Cox B.S. and Tuite M.F. (2001) The elimination of the yeast [PSI^+] prion by guanidine hydrochloride is the result of Hsp104 inactivation. Mol. Microbiol., **40,** 1357-1369.
43. Tuite M.F., Mundy C.R. and Cox B.S. (1981) Agents that cause a high frequency of genetic change from [PSI^+] to [psi^-] in Saccharomyces cerevisiae. Genetics, **98,** 691-711.
44. Eaglestone S.S., Ruddock L.W., Cox B.S. and Tuite M.F. (2000) Guanidine hydrochloride blocks a critical step in the propagation of the prion-like determinant [PSI^+] of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A, **97,** 240-244.
45. Jung G. and Masison D.C. (2001) Guanidine hydrochloride inhibits Hsp104 activity in vivo: a possible explanation for its effect in curing yeast prions. Curr. Microbiol., **43,** 7-10.
46. Wang K. (1982) Purification of titin and nebulin. Methods Enzymol., **85 Pt B,** 264-274.
47. Fritz J.D., Wolff J.A. and Greaser M.L. (1993) Partial titin cDNA sequence isolated from rabbit cardiac muscle RNA. J. Muscle Res. Cell Motil., **14,** 347-350.
48. Bang M.L., Centner T., Fornoff F., Geach A.J., Gotthardt M., McNabb M., Witt C.C., Labeit D., Gregorio C.C., Granzier H. and Labeit S. (2001) The complete gene sequence of titin, expression of an unusual approximately 700-kDa titin isoform, and its interaction with obscurin identify a novel Z-line to I-band linking system. Circ. Res., **89,** 1065-1072.
49. Derkatch I.L., Chernoff Y.O., Kushnirov V.V., Inge-Vechtomov S.G. and Liebman S.W. (1996) Genesis and variability of [PSI^+] prion factors in Saccharomyces cerevisiae. Genetics, **144,** 1375-1386.
50. King C.Y. (2001) Supporting the structural basis of prion strains: induction and identification of [PSI^+] variants. J. Mol. Biol., **307,** 1247-1260.
51. DePace A.H. and Weissman J.S. (2002) Origins and kinetic consequences of diversity in Sup35 yeast prion fibers. Nat. Struct. Biol., **9,** 389-396.
52. Wickner R.B., Taylor K.L., Edskes H.K., Maddelein M.L., Moriyama H. and Roberts B.T. (2000) Prions of yeast as heritable amyloidoses. J. Struct. Biol., **130,** 310-322.
53. Sambrook J., Fritsch E.E. and Maniatis T. (1989) Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor Press.
54. Greenbaum D., Jansen R. and Gerstein M. (2002) Analysis of mRNA expression and protein abundance data: an approach for the comparison of the enrichment of features in the cellular population of proteins and transcripts. Bioinformatics., **18,** 585-596.
55. Fernandez-Bellot E., Guillemet E., Ness F., Baudin-Baillieu A., Ripaud L., Tuite M. and Cullin C. (2002) The [URE3] phenotype: evidence for a soluble prion in yeast. EMBO Rep., **3,** 76-81.
56. Liu J.J., Sondheimer N. and Lindquist S.L. (2002) Changes in the middle region of Sup35 profoundly alter the nature of epigenetic inheritance for the yeast prion [PSI^+]. Proc. Natl. Acad. Sci. U. S. A, **99 Suppl. 4,** 16446-16453.
57. Valouev I.A., Kushnirov V.V. and Ter Avanesyan M.D. (2002) Yeast polypeptide chain release factors eRF1 and eRF3 are involved in cytoskeleton organization and cell cycle regulation. Cell Motil. Cytoskeleton, **52,** 161-173.
58. Chai Y., Koppenhafer S.L., Bonini N.M. and Paulson H.L. (1999) Analysis of the role of heat shock protein (Hsp) molecular chaperones in polyglutamine disease. J. Neurosci., **19,** 10338-10347.
59. Dul J.L., Davis D.P., Williamson E.K., Stevens F.J. and Argon Y. (2001) Hsp70 and antifibrillogenic peptides promote degradation and inhibit intracellular aggregation of amyloidogenic light chains. J. Cell Biol., **152,** 705-716.

10. PUBLICATIONS

The experimental data included in this report are described in the papers:

1. Kryndushkin D.S., Smirnov V.N., Ter-Avanesyan M.D. and Kushnirov V.V. (2002) Increased expression of Hsp40 chaperones, transcriptional factors, and ribosomal protein Rpp0 can cure yeast prions. J. Biol. Chem., **277**, 23702-23708

2. Kryndushkin D.S., Alexandrov I.M., Ter-Avanesyan M.D. and Kushnirov V.V. (2003) Yeast [PSI$^+$] Prion Aggregates Are Formed by Small Sup35 Polymers Fragmented by Hsp104. (**submitted** to The EMBO Journal).

The experimental data included in this report were presented at following conferences:

1. Kushnirov V.V., Kryndushkin D.S., Alexandrov I.M. and Ter-Avanesyan M.D. Sup35 prion aggregates are composed of small Sup35 oligomers fragmented by Hsp104. 2nd International Yeast Prion Meeting. Calistoga, CA, USA. August 25-28, 2002.

2. Kryndushkin D.S., Alexandrov I.M., Ter-Avanesyan M.D. and Kushnirov V.V. A novel method for analysis of the structure of prion protein aggregates in Saccharomyces cerevisiae. 3rd Congress of the Russian Biochemical Society, Sankt-Petersburg, Russia, 2002, p. 496.

11. SCIENTIFIC PERSONNEL

1. Michael D. Ter-Avanesyan, Ph.D, PI
2. Vitaly V. Kushnirov, Ph.D, CO-PI
3. Natalia V. Kochneva-Pervukhova, Ph.D
4. Igor A. Valouev
5. Maria B. Chechenova